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The excretion of uromodulin is modulated by the calcium-sensing receptor

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Uromodulin is produced in the thick ascending limb, but little is known about regulation of its excretion in urine. Using mouse and cellular models, we demonstrate that excretion of uromodulin by thick ascending limb cells is increased or decreased upon inactivation or activation of the calcium-sensing receptor (CaSR), respectively. These effects reflect changes in uromodulin trafficking and likely involve alterations in intracellular cyclic adenosine monophosphate (cAMP) levels. Administration of the CaSR agonist cinacalcet led to a rapid reduction of urinary uromodulin excretion in healthy subjects. Modulation of uromodulin excretion by the CaSR may be clinically relevant considering the increasing use of CaSR modulators.

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Uromodulin (Tamm-Horsfall protein), the most abundant protein excreted in normal urine, is synthesized in the cells lining the thick ascending limb (TAL) of the loop of Henle. Convergent studies indicate that uromodulin regulates salt transport and urinary concentration, protects against kidney stones and urinary tract infections, and plays roles in acute kidney injury and innate immunity. Furthermore, the *UMOD* gene coding for uromodulin has been associated with a spectrum of rare and common kidney diseases.[1]

Although the excretion of uromodulin in urine is known to fluctuate,[2] surprisingly little is known about the regulators of uromodulin abundance in the kidney and urine. The correlations between urinary levels of uromodulin and markers of tubular function[3,4] and the potential influence of arginine vasopressin[5] suggest that factors operating on the TAL may be involved. The CaSR is a G protein–coupled receptor expressed in the TAL, where it regulates paracellular Ca^{2+} reabsorption through mechanisms that include a dose-dependent inhibition of intracellular cAMP levels.[6,7] Inactivating or activating mutations of the *CASR* gene lead to rare hypercalcemia or hypocalcemia disorders, respectively.[8] The latter can be associated with Bartter-like syndrome, supporting the role of CaSR in maintaining the TAL function.[9] Whether the renal CaSR regulates uromodulin production and/or excretion by TAL cells is unknown.

Here we used mouse models with inactivating and activating mutations in the *Casr* gene, combined with pharmacologic agents and physiological stimuli, to demonstrate that chronic or acute modulation of the CaSR influences the urinary excretion of uromodulin.

We first verified that, in mouse kidney, *Umod* and *Casr* mRNAs are most abundantly expressed in the TAL (Figure 1a), with distinct apical (uromodulin) and basolateral (CaSR) distribution in that segment (Figure 1b). Immunoblotting on primary cultures of mouse TAL (mTAL) cells obtained from TAL segments[10] demonstrated that they endogenously express uromodulin and CaSR, NKCC2, and ROMK and excrete uromodulin into the apical medium (Figure 1c).

To examine whether the CaSR modulates uromodulin excretion *in vivo*, we analyzed mice with activating ($Casr^{Nuf/Nuf}$) and inactivating ($Casr^{BCH002/+}$) mutations in *Casr* (Supplementary Figure S1A and S1B). The activating *Casr* mutation p.Leu723Gln in $Casr^{Nuf/Nuf}$ mice (Supplementary Figure S1C and S1E)[11] results in significantly decreased

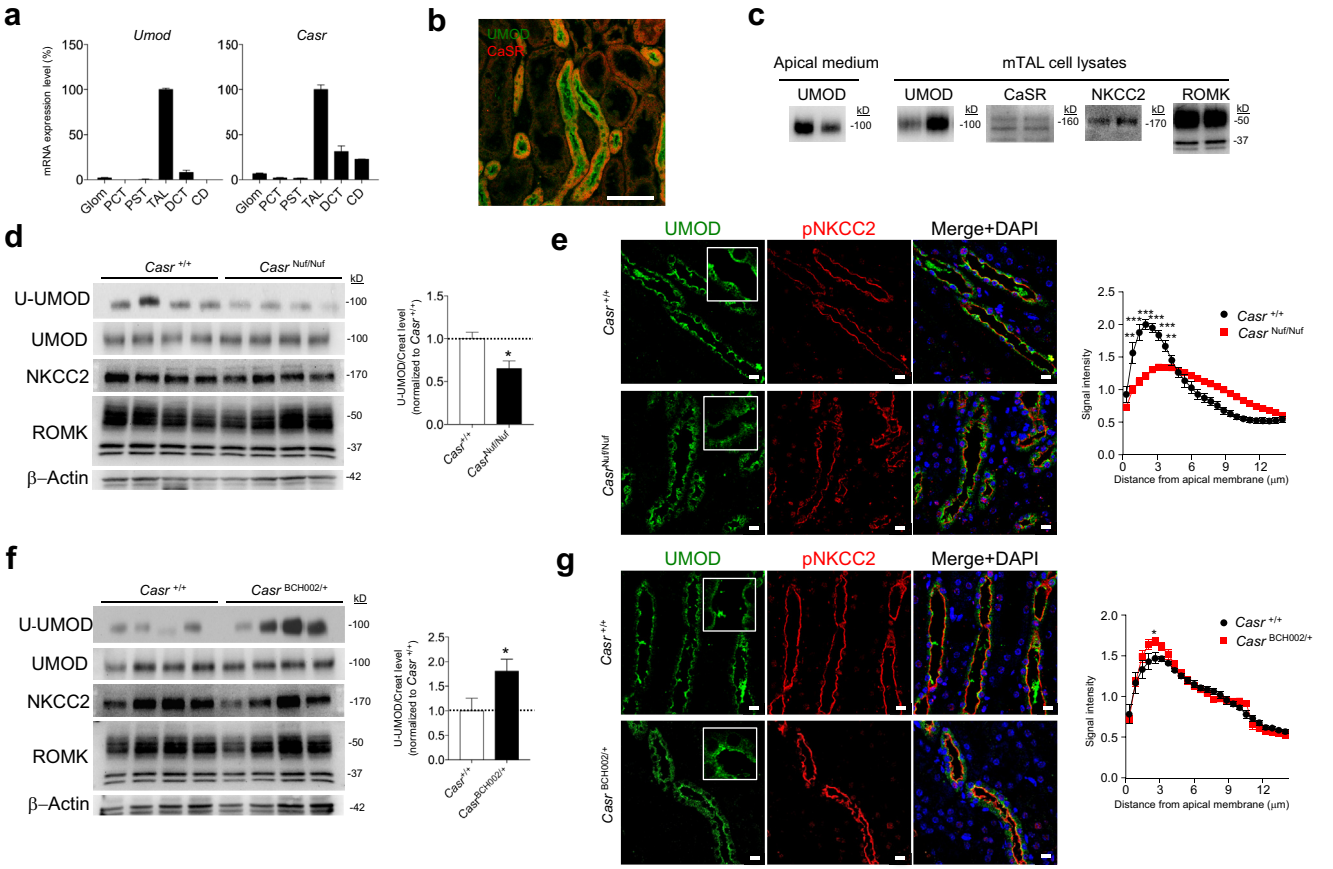

Figure 1 | Distribution of uromodulin and calcium-sensing receptor (CaSR) in the thick ascending limb (TAL) and the effect of CaSR mutations on uromodulin excretion. (**a**) Relative mRNA expression profile of *Umod* and *Casr* in microdissected glomeruli (Glom), proximal convoluted tubules (PCT), proximal straight tubules (PST), TAL, distal convoluted tubules (DCT), and collecting ducts (CD) from wild-type C57BL6J mice. TAL levels are taken as 100%. Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) expression was used for normalization. Bars indicate means ± SEM, $n = 6$. (**b**) Immunofluorescent staining showing the apical and basolateral distribution of uromodulin (green) and CaSR (red), respectively, in TAL profiles from mouse kidney. Bar = 100 μm. (**c**) Western blot analysis showing uromodulin excretion in the apical medium and endogenous expression of uromodulin, CaSR, NKCC2, and ROMK in mouse TAL (mTAL) cells. Each lane corresponds to the lysate from a distinct mTAL cell filter. (**d**) Western blot analysis of uromodulin in urine samples (U-UMOD) from $Casr^{Nuf/Nuf}$ mice harboring an activating *Casr* mutation and wild-type $Casr^{+/+}$ controls. Loading volume was adjusted according to creatinine concentration; each lane represents a different animal. The signal was quantified by densitometry (bars = means ± SEM) on $n = 4$ $Casr^{+/+}$ and $n = 5$ $Casr^{Nuf/Nuf}$ samples, $^{*}P < 0.05$, Student *t*-test. The protein expression levels of uromodulin, NKCC2, and ROMK in whole kidneys of $Casr^{Nuf/Nuf}$ versus $Casr^{+/+}$ mice are shown below the corresponding urine samples. β-Actin was used for loading control. (**e**) Immunofluorescent staining showing the subcellular distribution of uromodulin (green) and pNKCC2 (red) in kidney sections of $Casr^{+/+}$ and $Casr^{Nuf/Nuf}$ mice. Bar = 20 μm. The adjacent panel shows the line-plot analysis of uromodulin distribution in the $Casr^{+/+}$ (black) and $Casr^{Nuf/Nuf}$ (red) kidneys, with the apical membrane being defined by the pNKCC2 signal. Dots indicate means ± SEM for $n = 4$ mice. $^{**}P < 0.01$; $^{***}P < 0.001$, Student *t*-test. (**f**) Western blot analysis of U-UMOD from mice harboring an inactivating *Casr* mutation $Casr^{BCH002/+}$ and wild-type $Casr^{+/+}$ controls. Loading volume was adjusted according to creatinine concentration; each lane represents a different animal. The signal was quantified by densitometry (bars = means ± SEM) on $n = 4$ samples in each group, $^{*}P < 0.05$, Student *t*-test. The protein expression levels of uromodulin, NKCC2, and ROMK in whole kidneys of $Casr^{BCH002/+}$ versus $Casr^{+/+}$ mice are shown below the corresponding urine samples. β-Actin was used for loading control. (**g**) Immunofluorescent staining showing the subcellular distribution of uromodulin (green) and pNKCC2 (red) in mouse kidney sections of $Casr^{+/+}$ and $Casr^{BCH002/+}$ mice. Bar = 20 μm. The adjacent panel shows the line-plot analysis of uromodulin distribution in the $Casr^{+/+}$ (black) and $Casr^{BCH002/+}$ (red) kidneys, with the apical membrane being defined according to the pNKCC2 signal. Dots indicate means ± SEM for $n = 4$ mice. $^{*}P < 0.05$, Student *t*-test. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

plasma Ca^{2+} and Mg^{2+} levels, elevated plasma phosphorus levels, and lower urinary Ca^{2+}, Mg^{2+}, and phosphorus levels compared with $Casr^{+/+}$ mice (Supplementary Table S1). The $Casr^{Nuf/Nuf}$ mice excreted significantly less uromodulin in urine than did $Casr^{+/+}$ mice, despite similar uromodulin protein (Figure 1d and Supplementary Figure S2C) and slightly increased *Umod* mRNA levels in the kidneys (Supplementary Figure S2A). The lower urine levels were reflected by a less pronounced apical and more diffuse intracellular staining for uromodulin in TAL cells of the $Casr^{Nuf/Nuf}$ kidneys (Figure 1e). The *Casr* p.Ile859Asn inactivating mutation is causing early death in homozygous $Casr^{BCH002/BCH002}$ mice, likely because of decreased CaSR expression (Supplementary Figure S1D and S1F) and severe hyperparathyroidism and hypercalcemia.[12] Heterozygous $Casr^{BCH002/+}$ mice showed significantly increased blood Ca^{2+}

and decreased plasma phosphorus levels (Supplementary Table S1) and significantly higher levels of uromodulin in urine (Figure 1f), reflected by an enhanced apical staining for uromodulin in TAL cells (Figure 1g) compared with *Casr* $^{+/+}$ mice. Of note, uromodulin mRNA and protein expression levels (Supplementary Figure S2B and S2D) were unchanged in *Casr*$^{BCH002/+}$ versus *Casr*$^{+/+}$ kidneys.

We directly tested the effect of CaSR activation on uromodulin secretion by using well-established pharmacologic agents on mTAL cells. Treatment of mTAL cells with the CaSR agonist (calcimimetic) calindol (10 nM to 1 μM, 16 hours) induced a strong dose-dependent decrease in transepithelial voltage (Figure 2a), paralleled by a decrease in the excretion of uromodulin in the apical medium (Figure 2b) in the absence of detectable change in the expression of uromodulin in cell lysates (Supplementary Figure S3A). Short-term treatment (4 hours) of mTAL cells with 100 nM calindol significantly decreased the transepithelial voltage (Figure 2c) and the apical excretion of uromodulin (Figure 2d) in the absence of changes of uromodulin, NKCC2, and ROMK in corresponding cell lysates (Supplementary Figure S3B). Activation of the CaSR by high extracellular Ca^{2+} (3 mM, 6 hours) lowered transepithelial voltage (Figure 2e) and also resulted in a strong reduction of uromodulin excretion (Figure 2f) in the absence of changes in protein levels of uromodulin, NKCC2, and ROMK in cell lysates (Supplementary Figure S3D). The effects of extracellular Ca^{2+} on voltage and uromodulin excretion were abolished upon co-incubation with the specific CaSR antagonist NPS2143 (1 μM) (Figure 2e and f), which had no effect on uromodulin excretion in the absence of high basolateral Ca^{2+} concentrations (Supplementary Figure S3C). The potential link between CaSR signaling, uromodulin excretion, and intracellular cAMP levels is supported by the fact that calindol (which decreases uromodulin excretion: Figure 2b and d) prevented the 1-desamino-8d-arginine vasopressin (dDAVP)-induced rise of intracellular cAMP levels in mTAL cells (Figure 2g), whereas acute exposure of mTAL cells to dDAVP (100 nM, 4 hours) (Figure 2h) and to cAMP (50 μM, 4 hours) (Figure 2i) increased uromodulin excretion.

To verify the translational value of the link between CaSR and uromodulin excretion, we tested the effect of a single dose (60 mg) of the calcimimetic drug cinacalcet in healthy subjects (Figure 2j and k). As expected, administration of cinacalcet induced a rapid increase (+266%) of urinary calcium excretion that was paralleled by a decrease (–18%) in urinary excretion of uromodulin.

The data presented here demonstrate for the first time that urinary excretion of uromodulin is modulated by the CaSR operating in the basolateral membrane of the TAL. The use of primary mTAL cells with endogenous expression of uromodulin highlights the value of this system to investigate the biology of uromodulin. The link between CaSR activity and excretion of uromodulin is evidenced by (i) decreased or increased urinary uromodulin levels in mice harboring activating or inactivating mutations of the *Casr*, respectively; (ii) decreased uromodulin excretion after activation of the CaSR in mTAL cells by a specific CaSR agonist; (iii) a strong reduction in uromodulin excretion after activation of the CaSR in mTAL cells by high extracellular Ca^{2+}; (iv) abolition of the effects of high extracellular Ca^{2+} upon treatment of mTAL cells with the CaSR antagonist NPS2143; and (v) a significant decrease in uromodulin excretion after a single dose of cinacalcet in healthy subjects.

The fact that the changes in uromodulin excretion are reflected by modifications of its intracellular distribution rather than changes in total mRNA and protein levels suggests that the CaSR may regulate the trafficking of uromodulin. Activation of the CaSR decreases intracellular cAMP levels, resulting from both the inhibition of its production and the stimulation of its degradation.[7,13] The cAMP/protein kinase A pathway is involved in the trafficking of many proteins in tubular cells, including NKCC2 in the TAL and aquaporin-2 and epithelial sodium channel in the collecting ducts. Our data show that mTAL cells respond to dDAVP and cAMP by increasing the apical excretion of uromodulin; conversely, activation of the CaSR by calindol decreases uromodulin excretion and prevents the dDAVP-induced rise in cAMP in mTAL cells. Taken together, these observations support a role for CaSR-dependent changes in intracellular cAMP levels in uromodulin trafficking. The specific production of uromodulin in TAL cells and the association of urine levels of uromodulin with markers of tubular function[3,4] and genetic variants in *KCNJ1*/ROMK[2] suggest a cell-autonomous regulation. In fact, changes of uromodulin excretion induced by CaSR modulators in mTAL cells were paralleled by changes in the transepithelial voltage. The latter is predominantly K^+ dependent, because the luminal recycling of K^+ via the apical ROMK channel generates the lumen-positive voltage in TAL. Extracellular Ca^{2+} was shown to generate cytochrome P-450 metabolites that inhibit ROMK activity, likely via CaSR signaling.[14] Future studies should decipher the mechanisms linking CaSR, ROMK, and uromodulin secretion, including a potential role of the serine protease hepsin.[15]

In summary, the activation of CaSR modulates the excretion of uromodulin in the urine, probably through post-translational control of uromodulin trafficking and cAMP levels in TAL cells. The possibility of modulating urinary levels of uromodulin may be clinically relevant, considering the multifaceted role of this protein in health and disease and the increasing use of pharmacologic modulators of the CaSR.

SHORT MATERIALS AND METHODS

Mouse models

Studies were conducted in C57BL6J mice and *Casr*$^{Nuf/Nuf}$ (11) and *Casr*$^{BCH002/+12}$ mice, using well-established protocols.[16]

Human studies

Healthy subjects gave informed consent to test the effect of a single dose of cinacalcet, 60 mg, on the excretion of uromodulin and calcium in urine.

Urine and blood chemistry

Urine and blood parameters were determined as described.[16,17]

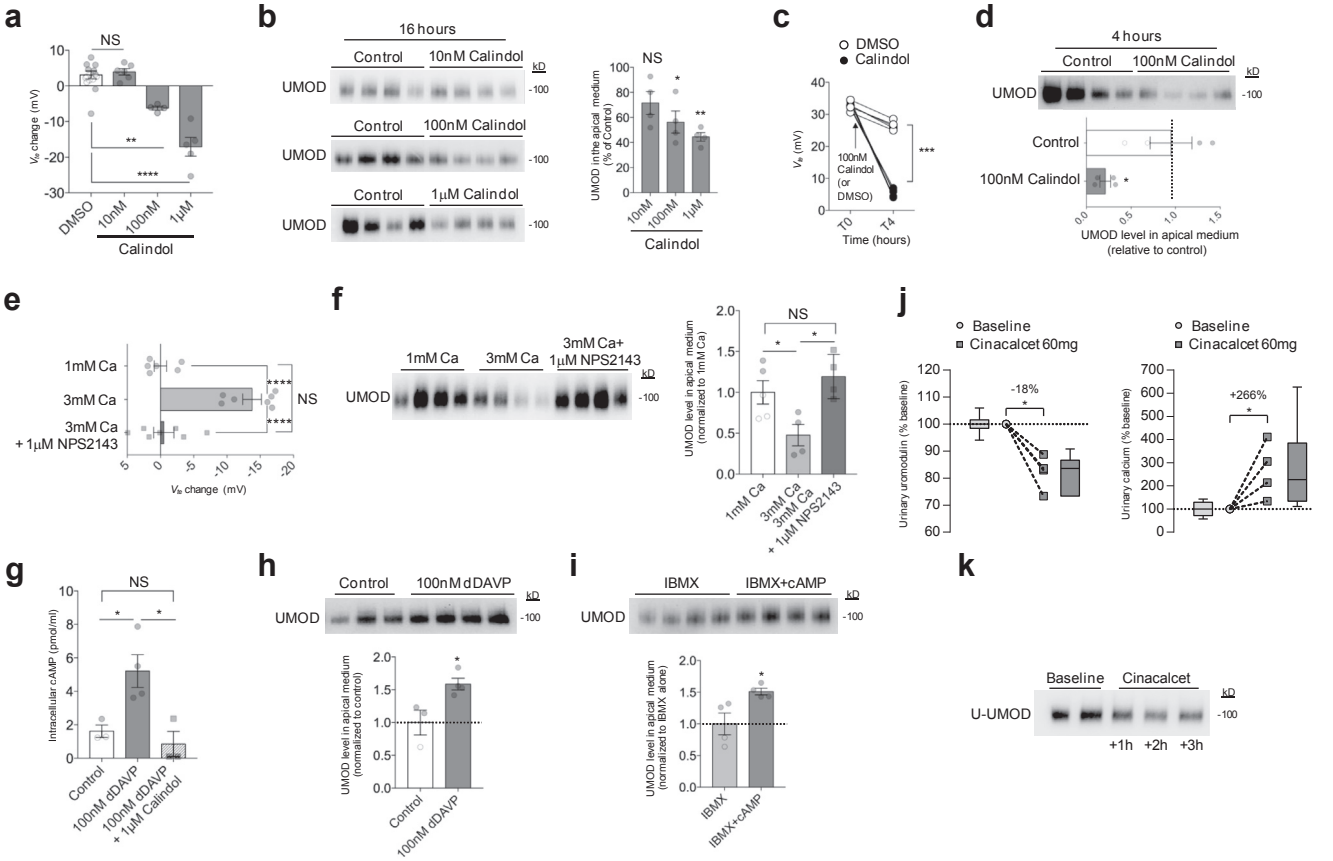

Figure 2 | Modulating calcium-sensing receptor (CaSR) activity influences uromodulin excretion in mouse thick ascending limb (mTAL) cells and healthy subjects. (**a–d**) Effects of calindol on mTAL cells. (**a**) Changes in transepithelial voltage (V_{te}) of mTAL cells treated with dimethylsulfoxide (DMSO) (white bar, $n = 12$) and the CaSR agonist calindol (10 nM, 100 nM, or 1 μM to the basolateral side, $n = 4$–6 for each condition) for 16 hours. Bars indicate means ± SEM. Analysis of variance (ANOVA): $P < 0.0001$; Tukey's multiple comparisons test: NS, not significant; $^{**}P < 0.01$; $^{****}P < 0.0001$. Only comparisons with DMSO are shown. (**b**) Western blot analysis and relative uromodulin levels in the apical medium of mTAL cells treated with DMSO and 10 nM to 1 μM calindol for 16 hours (basolateral side). Each lane corresponds to 1 different mTAL cell filter ($n = 4$ per condition). Equal volumes were loaded. Quantification by densitometry (bars indicate means ± SEM). (**c**) V_{te} of mTAL cells treated with DMSO (open symbols) or 100 nM calindol (black symbols) for 4 hours on the basolateral side ($n = 5$ for each condition), with (**d**) corresponding Western blot analysis for uromodulin levels in the apical medium of mTAL cells. Each lane corresponds to a different mTAL cell filter. Quantification of the signal by densitometry (bars indicate means ± SEM), with values expressed relative to control. (**b–d**) NS, not significant; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, Student *t*-test. (**e,f**) Effects of extracellular calcium and NPS2143 on mTAL cells. (**e**) Changes in V_{te} of mTAL cells exposed to variable extracellular Ca^{2+} concentrations (1 mM, $n = 6$; 3 mM, $n = 7$), and 3 mM Ca^{2+} with 1 μM NPS2143 to the basolateral side ($n = 7$) for 6 hours. (**f**) Representative Western blots for uromodulin levels in apical medium of mTAL cells treated with 1 mM and 3 mM Ca^{2+} medium, and 3 mM Ca^{2+} with 1 μM NPS2143 at the basolateral side for 6 hours. Each lane corresponds to a different mTAL cell filter. Quantification of the signal by densitometry (bars indicate means ± SEM), relative to 1 mM Ca^{2+} ($n = 4/5$ for each condition). ANOVA: $P < 0.0001$ for (**e**) and $P = 0.0140$ for (**f**). Tukey's multiple comparisons test: NS, not significant; $^{*}P < 0.05$; $^{****}P < 0.0001$. (**g–i**) Effects of 1-desamino-8d-arginine vasopressin (dDAVP) and cyclic adenosine monophosphate (cAMP) on mTAL cells. (**g**) Intracellular cAMP levels (pmol/ml of lysate) in mTAL cells treated with DMSO (control), 100 nM dDAVP for 1 hour, and 100 nM dDAVP with 1 μM calindol for 1 hour, all treatments to the basolateral side ($n = 3/4$ for each condition). Each value represents an independent filter. Bars indicate means ± SEM. ANOVA: $P = 0.0129$; Tukey's multiple comparisons test: NS, not significant; $^{*}P < 0.05$. Western blot analysis of uromodulin excretion levels in apical medium of mTAL cells treated with (**h**) DMSO (control) versus 100 nM dDAVP to the basolateral side ($n = 3/4$) and (**i**) with 3-isobutyl-1-methylxanthine (IBMX) alone (50 μM) versus IBMX + cAMP (50 μM each) ($n = 4$) for 4 hours. Quantification by densitometry (bars indicate means ± SEM) below the corresponding blots; values are expressed relative to control or IBMX. $^{*}P < 0.05$; Student *t*-test. (**j,k**) Effect of cinacalcet on urinary uromodulin excretion in humans. (**j**) Relative changes in urinary uromodulin (left) and calcium (right) levels in healthy volunteers after a single oral ingestion of 60 mg cinacalcet. Urine samples were collected at baseline and 1 to 2 hours after ingestion of cinacalcet. Uromodulin and calcium levels were normalized to urinary creatinine (mg/g creatinine) and are shown relative (in percent) to baseline levels. Symbols represent mean values before and after cinacalcet administration for a given volunteer ($n = 4$, linked by dashed lines). Box and whiskers (minimum to maximum) represent the distribution of the individual values before and after administration of cinacalcet. $^{*}P < 0.05$; Mann-Whitney test. (**k**) Western blot analysis for uromodulin in urine samples (U-UMOD) from a representative subject at baseline and after a single dose of 60 mg cinacalcet. Loading volume was adjusted according to creatinine concentration for each sample.

Primary cell culture of TAL cells

Primary cell cultures of mTAL cells were obtained and cultured as described.[10] Confluent mTAL monolayers were exposed (basolateral side) to calindol,[18] NPS2143,[19] dDAVP, and 3-isobutyl-1-methylxanthine (IBMX) alone or with cAMP, as detailed in the supplementary material. Electrophysiology was performed as described.[10] The cAMP levels were determined with the DetectX Cyclic AMP enzyme immunoassay kit (Arbor Assays, Ann Arbor, MI).

Gene and protein expression analyses

Gene and protein expression analyses were performed using primers, conditions, and antibodies listed in the Supplementary Material.

Statistics

Data are presented as mean ± SEM. Two-tailed unpaired Student *t*-test, Mann-Whitney test, or analysis of variance with Tukey's multiple comparison tests were used for the statistical analysis, as indicated.

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Supplementary Materials and methods.
Figure S1. Description of the *Casr* mutant mice.
Figure S2. Transcriptional and protein expression profiles in kidneys of the *Casr* mutant mice.
Figure S3. Effect of calcium-sensing receptor modulators, 1-desamino-8d-arginine vasopressin (dDAVP), and cyclic adenosine monophosphate (cAMP) on uromodulin expression in mTAL cells.
Table S1. Urine and blood parameters in *Casr* mutant mice.
Table S2. List of primers.
Supplementary material is linked to the online version of the paper at www.kidney-international.org.

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